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Occurrence and Expression of Luminescence in *Vibrio cholerae*▽

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Several species of the genus *Vibrio*, including *Vibrio cholerae*, are bioluminescent or contain bioluminescent strains. Previous studies have reported that only 10% of *V. cholerae* strains are luminescent. Analysis of 224 isolates of non-O1/non-O139 *V. cholerae* collected from Chesapeake Bay, MD, revealed that 52% (116/224) were luminescent when an improved assay method was employed and 58% (130/224) of isolates harbored the *luxA* gene. In contrast, 334 non-O1/non-O139 *V. cholerae* strains isolated from two rural provinces in Bangladesh yielded only 21 (6.3%) luminescent and 35 (10.5%) *luxA*$^+$ isolates. An additional 270 clinical and environmental isolates of *V. cholerae* serogroups O1 and O139 were tested, and none were luminescent or harbored *luxA*. These results indicate that bioluminescence may be a trait specific for non-O1/non-O139 *V. cholerae* strains that frequently occur in certain environments. Luminescence expression patterns of *V. cholerae* were also investigated, and isolates could be grouped based on expression level. Several strains with defective expression of the *lux* operon, including natural K variants, were identified.

Vibrio cholerae is an autochthonous marine and estuarine bacterium and the causative agent of cholera, a severe and life-threatening human diarrheal disease. There are more than 200 different serogroups, but only the O1 and O139 serogroups are responsible for the epidemic clinical disease, although several of the non-O1/non-O139 serogroups have been responsible for less severe diarrheal disease outbreaks.

Bioluminescence is a property possessed by several marine bacteria, mostly members of the family *Vibrionaceae*, including subpopulations of environmental isolates of *V. cholerae* (10, 13). Luminescence is controlled by the *lux* operon and is regulated in a cell density-dependent manner, termed quorum sensing or autoinduction, a response in target gene expression when extracellular signal molecules, called autoinducers, reach a critical concentration. Bassler et al. (3) reported that *V. cholerae* O1, as well as *Vibrio anguillarum*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Vibrio natriegens* were able to induce bioluminescence in *Vibrio harveyi* reporter strains, indicating that *lux* regulatory genes are present. The genomic sequence of *V. cholerae* O1 El Tor N16961 includes several homologues of the well-characterized *V. harveyi lux* regulatory system (5), including *luxO*, *-P*, *-Q*, *-R (hapR* [6]), *-S*, *-U*, and *-N* (*cqsS* [9]). Notably missing from the genome sequence of *V. cholerae* N16961 is the *lux*"structural" operon, comprised of five essential genes, *luxCDABE*.

Twenty years ago, West et al. (13) conducted a numerical taxonomic study of vibrios and found that 11/115 (10%) strains of *V. cholerae* were bioluminescent, as determined by examining colonies on agar plates in the dark after a period of adjustment. Palmer and Colwell (10) reexamined 62 of the 115 nonluminescent strains from the study by assaying 6-h LB broth cultures using both visual examination and liquid scintillation counting. They found that 5% (3/62) were dimly luminescent (by visual examination) and 16% (10/62) emitted "low-level" light (detectable solely by the liquid scintillation counting). Additionally, 56% (35/62) hybridized to a *Vibrio fischeri luxA* probe.

In this study, we developed a method that allowed a more sensitive assay for expression of luminescence in *Vibrio cholerae*. Additionally, we employed a *V. cholerae*-specific genetic screen for the *lux* gene *luxA* to confirm luminescent strains identified by the expression assay. We reevaluated 47 previously characterized *V. cholerae* strains (10) and screened 224 environmental *V. cholerae* isolates from Chesapeake Bay, MD; 400 environmental and 156 clinical *V. cholerae* isolates from Bangladesh; and an additional 48 *V. cholerae* clinical isolates from various sources for luminescence to determine the natural occurrence of this trait. Luminescence assay results were compared to results of either *luxA* PCR or dot blot hybridization to assess the sensitivity and specificity of the assay. Luminescence expression levels of selected groups of *V. cholerae* were also investigated in detail, with isolates characterized according to luminescence expression.

MATERIALS AND METHODS

Bacterial strains. A total of 875 strains of *V. cholerae* were characterized for luminescence in this study. Of this total, 605 were from non-O1/non-O139 serogroups, 164 were serogroup O1, and 106 were serogroup O139. The O1/O139 serogroup strains included clinical and environmental isolates. Additionally, 17 *V. mimicus* strains were used. The strains were divided into five groups (Table 1). Selected strains, i.e., UM4157, UM4089, UM4086, UM4057, and UM4102, from group I were employed to develop the luminescence bioassay used in this study, since they had been previously characterized in two separate studies (10, 13).

Luminescence bioassay. Test strains were subcultured from −80°C frozen stock onto agar plates and incubated overnight at 30°C. Single colonies were inoculated into fresh broth medium and incubated overnight at 30°C with aeration (200 rpm). The overnight culture was diluted to 1:500 or 1:1,000 into

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TABLE 1. Bacterial strains used in this study

Group	Species	Serogroup	No. of strains	Source (reference[s])
I	*V. cholerae*	Non-O1/non-O139	47	Previously characterized collection from various geographical locations (10, 13)
II	*V. cholerae*	Non-O1/non-O139	224	Chesapeake Bay, 1998–1999 (7)
III	*V. cholerae*	Non-O1/non-O139	334	Environmental and clinical isolates, ICDDR,B,[a] 2004–2005 (1)
		O1	40	
		O139	26	
IV	*V. cholerae*	O1	94	Clinical isolates, ICDDR,B, 1997–2000 (11)
		O139	62	
V	*V. cholerae*	O1	30	University of Maryland
		O139	18	
VI	*V. mimicus*		17	University of Maryland

[a] ICDDR,B, International Centre for Diarrhoeal Disease Research, Bangladesh.

125-ml flasks or 16- by 125-mm culture tubes containing fresh medium and incubated at 30°C with aeration (200 rpm). Luminescence was measured at successive time points using an LB96P luminometer (Berthold, Oak Ridge, TN). Relative light units (RLU) are defined here as the number of events observed per second per 100 μl of culture. The instrument gave a background measurement of 20 RLU when uninoculated medium or *Escherichia coli* DH5α was assayed; therefore, a mean luminometer reading of 30 RLU or higher was considered positive. Simultaneously, culture density was measured using optical density at 600 nm (OD_{600}), employing a DU640 UV/visible spectrophotometer (Beckman-Coulter, Fullerton, CA).

Five media were tested for producing optimal luminescence in *V. cholerae*: Luria-Bertani medium plus 1% NaCl (LBN), luminous medium (LUM) (ATCC medium 731), autoinducer bioassay medium (AB) (2); *Photobacterium* medium (PB), and Marine broth 2216 (MB) (Difco, Detroit, MI).

Different air-to-sample volume ratios were tested to determine the optimal ratio for the luminescence bioassay at a constant shaking speed of 200 rpm. Culture tubes (16 by 125 mm) were filled with 2.5-, 5-, or 10-ml sample volumes, and 150-ml Erlenmeyer flasks were filled with 75, 50, 25, or 10 ml of sample. Three representative strains from group I, i.e., *V. cholerae* UM4057, UM4086, and UM4102, were assayed in triplicate.

***V. cholerae luxA* PCR.** To test the sensitivity of the luminescence assay, *luxA* PCR amplification or *luxA* dot blot hybridization was performed on genomic DNA extracted from each *V. cholerae* isolate used in this study. For groups I and IV, *luxA* PCR was used. A new, highly specific *V. cholerae luxA* PCR primer pair (VCluxA108F/757R), 5′-CGAAGCGGTTTGGTTGCTA-3′ and 5′-CGGGTA GCATTGACGTAGGA-3′, which amplify a 650-bp fragment, were designed. Confirmatory *luxA* PCR, using *Taq* polymerase (Promega, Madison, WI), was performed according to standard protocols.

***V. cholerae luxA* dot blot hybridization.** For groups II, III, and V, *luxA* dot blot hybridization was performed. DNA was extracted using the DNeasy tissue kit (Qiagen) and dot blotted onto MagnaCharge membranes (Osmonics, Westborough, MA) according to the manufacturer's instructions. A 650-bp *luxA* probe labeled with digoxigenin (DIG)-dUTP was produced, using the PCR DIG probe synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany) and primers VCluxA108F/757R (described above). The PCR product was analyzed by agarose gel electrophoresis. Hybridization was performed using the DIG detection starter kit II (Roche Molecular Biochemicals). Dot blots were hybridized at 45°C overnight. Autoradiography was performed for 20 min at 25°C.

Luminescence expression. Luminescence expression levels of groups I, II, and III were analyzed to determine a reference, or "normal," intensity of expression (10,001 to 100,000 RLU) and to identify "dark" (*luxA*$^+$ and nonluminescent; 0 RLU), "dim" (1 to 100 RLU), and "defective" (101 to 10,000 RLU) strains. Luminescence activity is reported as the maximal expression value over at least four time points and at least three replicates, in all cases. Early investigations determined a concise window for measurement, based on culture density. *V. harveyi* ATCC 14126, *V. fischeri* ATCC 7744, and *V. cholerae* biotype Albensis ATCC 14547 served as positive controls and *E. coli* DH5α and uninoculated medium as negative controls.

Growth and luminescence kinetics. Growth and luminescence curves were measured for three strains of *V. cholerae* to compare the luminescence kinetics of *V. cholerae* to those of *V. harveyi* and *V. fischeri*. Luminescence, culturable plate counts on Marine agar 2216 plates, and OD_{600} were measured every hour for a minimum of 24 h. All experiments were performed at least in triplicate.

RESULTS

Luminescence bioassay for *V. cholerae*. The results from two previous studies involving *V. cholerae* indicate that not all strains express luminescence at normal levels and that in fact some strains exhibit "low-level" light production (10, 13). For *V. cholerae*, a luminescence bioassay must be able to identify all luminescent strains regardless of the expression level, and therefore the assay must have a wide dynamic range of detection. The bioassay method developed in this study for luminescence of *V. cholerae* was based on a previously described autoinducer bioassay, in which cross-species induction of a *V. harveyi* reporter strain was investigated (3). Optimization of our bioassay was achieved using five *V. cholerae* strains from group I (UM4057, UM4086, UM4089, UM4102, and UM4157), since they were previously characterized as exhibiting defective luminescence expression (10, 13). The five strains were grown in five different media and assayed for expression of luminescence over a 10-h time period, using the new bioassay method. For each of the five strains, expression of luminescence was significantly higher in MB (Table 2), indicating that this medium is superior when employed to determine luminescence expression by *V. cholerae* and using the luminescence bioassay developed in this study. On average, results obtained when MB was employed were sevenfold higher than those with any of the other four media. Additionally, none of the strains were luminescent when grown in LUM or PB,

TABLE 2. Expression of luminescence by five strains of *V. cholerae* in five media, examined over a 10-hour time period

V. cholerae strain	RLU in:				
	AB	LUM	LB	MB	PB
UM4157	10	0	20	62	0
UM4102	95	0	893	5,529	0
UM4089	9,286	5,574	44,081	105,984	3,838
UM4086	30	0	21	1,718	0
UM4057	49	0	144	2,176	0
Mean	1,894	1,115	9,032	23,094	768

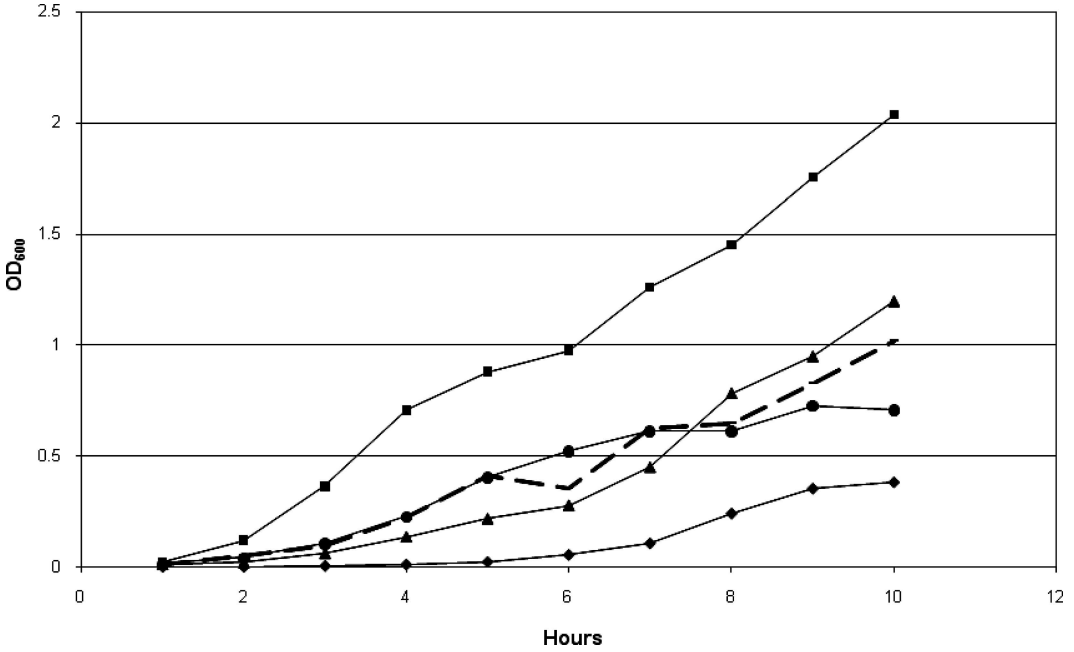

FIG. 1. Growth of *V. cholerae* UM4089 in five media during incubation for 10 h. Squares, LBN; triangles, LUM; diamonds, AB; circles, PB; dashed line, MB.

except *V. cholerae* UM4089, which was luminescent in all media employed in this study (Table 2). For strains grown in MB, expression of luminescence increased rapidly at ca. 4 h postdilution and stabilized at 5 to 6 h postdilution, except in the case of *V. cholerae* UM4157, which stabilized at 8 h postdilution. These results indicate that luminescence should be measured over a range of time points using this bioassay.

To determine whether differences in cell density could account for the differences in luminescence expression in the different media, culture density (OD_{600}) was simultaneously measured during the luminescence assay. Results for *V. cholerae* UM4089 are presented in Fig. 1 and are representative of those for the five strains tested. All five strains grew to a higher cell density in LBN than in the other media employed. Cultures grown in MB, LUM, and PB grew to comparable cell densities, and at a comparable growth rates, while the AB cultures exhibited the lowest cell density and growth rate. The culture density at time of maximum luminescence expression, as measured by OD_{600}, ranged from 0.6 to 0.8.

Evaluation of bioassay. Using the optimized protocol, *V. cholerae* strains of group I were assayed for expression of luminescence using the new bioassay and for the presence of *luxA* by the new *luxA* PCR protocol, for comparison with the results obtained by Palmer and Colwell (10). Table 3 shows that 8 of the 47 strains (17%) were luminescent according to the new bioassay. As expected, these luminescent strains also possessed *luxA*, as confirmed by PCR using *V. cholerae luxA* primers. Additionally, four of the nonluminescent strains, *V. cholerae* UM4071, UM4072, UM4075, and UM4082, were positive by *luxA* PCR, for a total of 12, or 26%. Using the results from the new *luxA* PCR screen, the new bioassay had a sensitivity of 67% and a specificity of 100%. The previous assay (10) also had a sensitivity of 67%, but its specificity was only 86%. RLU values from the two studies cannot be directly compared, since they are affected by protocol factors such as sampling vessel and time of reading.

Survey of luminescence in *V. cholerae*. The 224 environmental *V. cholerae* isolates from the Chesapeake Bay (group II) were assayed using the new bioassay for luminescence expression, and 116 were positive (52%).

Dot blots of DNAs extracted from the 224 isolates were probed using a DIG-labeled 650-bp *V. cholerae luxA* probe. Fifty-eight percent (130/224) were positive for *luxA* by hybridization. In addition to the 116 strains that were luminescent by assay, 14 strains were positive for *luxA* but did not express luminescence. These 14 strains were retested, with the same results.

Similarly, group III, IV, and V isolates were assayed for luminescence expression and screened for *luxA* by PCR or dot blot hybridization. For group III, none of the O1 or O139 serogroup isolates (0/40 and 0/26, respectively) were luminescent. Among the non-O1/non-O139 strains from group V, 6% (21/334) were luminescent and 11% (35/334) harbored the *luxA* gene. For groups IV and V, 0% (0/48 and 0/156, respectively) were luminescent or harbored *luxA*. Additionally, 17 strains of *V. mimicus* were tested, and all were negative by the bioassay and the genotypic screen.

Distribution of luminescence expression among *V. cholerae*. Groups II and III were categorized by level of maximal luminescence expression in an effort to define reference luminescence expression levels for *V. cholerae* (Fig. 2). The values of maximum luminescence expression obtained for each isolate, which were measured over a period of 4 h, are shown grouped, using a log-scale distribution. This was done because of the wide dynamic range of luminescence expression that was measured. Of 116 luminescent strains isolated

TABLE 3. Phenotypic expression and genotypic confirmation of luminescence in 47 previously characterized strains of *V. cholerae*

V. cholerae strain	RLU[a]			*V. cholerae luxA* PCR[c,d]
	Low-level light emission[b]	Luminescence[c] in:		
		LB	MB	
UM4052	−	−	−	−
UM4054	−	−	−	−
UM4055	−	−	−	−
UM4056	6.9	−	15	+
UM4057	10.1	5	2,101	+
UM4058	−	−	−	−
UM4060	−	−	−	−
UM4065	−	−	−	−
UM4068	−	−	−	−
UM4069	−	−	−	−
UM4070	−	−	−	−
UM4071	−	−	−	+
UM4072	−	−	−	+
UM4073	−	−	−	−
UM4075	−	−	−	+
UM4078	−	−	−	−
UM4079	−	−	−	−
UM4080	−	−	−	−
UM4082	−	−	−	+
UM4083	−	−	−	−
UM4086	9.5	6	1,167	+
UM4089	11.8	9,264	209,630	+
UM4091	7.0	−	18	+
UM4092	−	−	−	−
UM4093	−	−	−	−
UM4094	−	−	−	−
UM4096	−	−	−	−
UM4097	6.0	−	−	−
UM4098	−	−	−	−
UM4100	8.8	−	−	−
UM4102	11.1	164	18,471	+
UM4103	7.1	−	19	+
UM4104	6.8	−	−	−
UM4105	5.6	−	−	−
UM4157	10.0	−	412	+
UM4178	6.3	−	−	−
UM4180	−	−	−	−
UM4181	−	−	−	−
UM4182	−	−	−	−
UM4183	−	−	−	−
UM4184	−	−	−	−
UM4187	−	−	−	−
UM4188	−	−	−	−
UM4189	−	−	−	−
UM4195	−	−	−	−
UM4196	−	−	−	−
UM4217	−	−	−	−

[a] −, no signal.
[b] From reference 10.
[c] From this study.
[d] + or −, presence or absence of amplicon by gel electrophoresis.

from the Chesapeake Bay, 85.3% were luminescent at a level of 10,000 to 100,000 RLU. This range of expression is referenced as normal, since it represents the highest percentage of luminescent isolates from either group and contains the mean expression of all luminescent isolates of both groups. Among luminescent strains from the Chesapeake Bay (group II), 15% revealed a defect in luminescence expression (Fig. 2A), with 11 isolates (9.5%) luminescent at 10% of normal, 5 isolates (4.3%) luminescent at 1% of normal, and 1 strain expressing luminescence at below 0.1% of normal. Two of the 16 defective strains of group II are accounted for by the fact that they were at low cell density at the time luminescence was measured (Fig. 3A). These two grew much more slowly than the other 222 isolates and did not reach an OD_{600} of above 0.5 after 12 h of growth.

Of the luminescent isolates from Bangladesh, 52% (11/21) of the non-O1/non-O139 isolates from group III expressed light at normal levels (Fig. 2B). Interestingly, 29% (6/21) expressed light in the 0- to 100-RLU range (0.1% of normal). Three isolates expressed light below normal but above 0.1%. Also, one strain was luminescent at a level above normal for *V. cholerae*, approaching that of *V. fischeri* and *V. harveyi*. Analysis of luminescence expression versus cell density revealed that two of the six "dim" strains are accounted for by slow growth (Fig, 3B).

In addition to visibly luminescent strains, groups II and III both contained "dark" strains, i.e., strains containing at least one gene from the *lux* operon but emitting no visible light. In group II there were 14 such isolates (6.3%), and in group III there were also 14 (4.2%).

The 12 luminescent *V. cholerae* strains from group I were also characterized by luminescence expression. Strains UM4071, UM4072, UM4075, and UM4086 are completely "dark" strains (nonluminescent but harboring *luxA*). Strains UM4056, UM4091, and UM4103 are "dim" strains (0.1 to 1% of normal), with light expression that is barely detectable (~20 RLU). Strain UM4102 expressed luminescence at a normal level, and UM4089 expressed luminescence at a level above normal, or a "super-bright" level.

Growth curve and luminescence kinetics. The growth and luminescence for three strains of luminescent *V. cholerae* from group I (UM4086, UM408, and UM4103) with different levels of maximal luminescence expression, as well as one control strain of *V. fischeri* (MJ-100) and *V. harveyi* (BB-120) were assayed over a 24-h period (Fig. 4) to determine how expression of luminescence in *V. cholerae* compared to that in the other two species; *V. fischeri* and *V. harveyi* were assayed longer since their growth was slower (Fig. 4D and E). The three strains of *V. cholerae* expressed luminescence during mid- to late-exponential growth, consistent with an autoinduction-controlled phenotype (Fig. 4A to C).

The two strains *V. fischeri* MJ-100 and *V. harveyi* BB-120 were significantly brighter in luminescence than the three strains of *V. cholerae*. The brightest strain of luminescent *V. cholerae*, UM4089, had a maximal light expression value of 10^5 RLU (Fig. 4A), while the controls strains were brighter by 1 log unit (Fig. 4D and E). In addition, luminescence decay was more rapid among the luminescent *V. cholerae* strains than in *V. fischeri* and *V. harveyi*.

The three strains of luminescent *V. cholerae* revealed a noteworthy trend, i.e., that initiation of luminescence determined the maximum expression level of luminescence. That is, when initiation of luminescence occurred earlier in the growth curve (approximately mid-log phase), luminescence expression was higher. For example, *V. cholerae* UM4089 represents a "super-bright" luminescent *V. cholerae* strain. For this strain, the onset of luminescence expression occurred at a cell density of 1.5×10^7 cells/ml or an OD_{600} of 0.0421 (Fig. 4A). For *V. cholerae* UM4086, which is a defective strain, the onset of luminescence

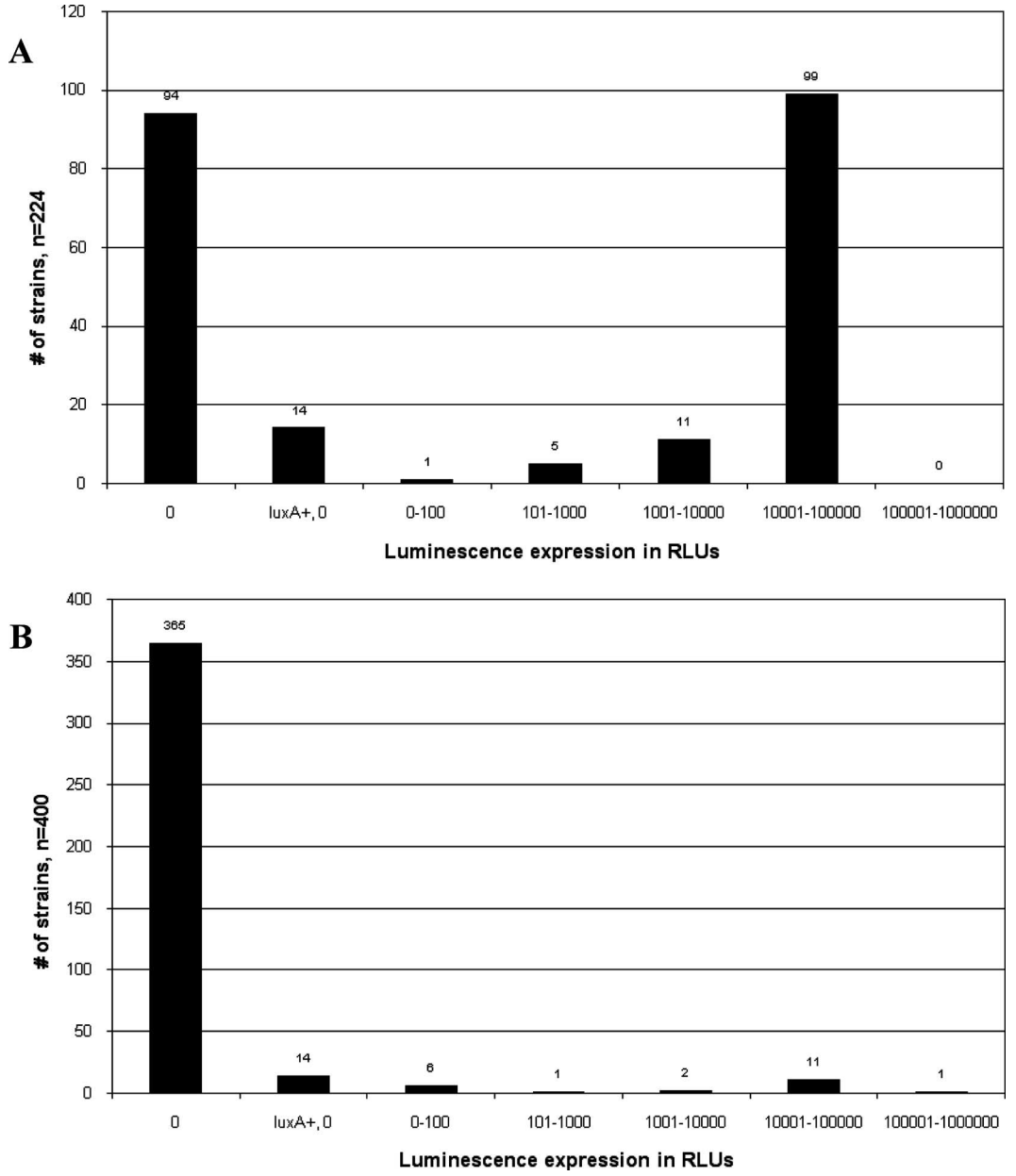

FIG. 2. Distribution of luminescent *V. cholerae* by luminescence expression level. (A) Chesapeake Bay (group II); (B) Bangladesh (group V). RLU is defined in Materials and Methods. The expression level of 15 RLU is the lowest considered luminescent.

expression occurred later in the growth cycle (approximately mid- to late-log phase) than for UM4089, at a higher cell density (7.6×10^7 cells/ml; OD_{600} of 0.1937) (Fig. 4B). In *V. cholerae* UM4103, a "dim" strain, the onset of luminescence expression was even later (approximately late-log phase), at a cell concentration of 1.1×10^8 cells/ml or an OD_{600} of 0.2177. The onset of luminescence expression is early in both *V. fischeri* and *V. harveyi* (Fig. 4D and E), translating into a high luminescence expression level. The growth and luminescence kinetic profile for *V. cholerae* biovar Albensis were also examined and found to be similar to those for *V. cholerae* UM4089 (not shown).

DISCUSSION

Twenty years ago, before molecular genetic methods were available, standard procedures for assaying the bioluminescence of *V. cholerae* employed visual determination of a culture streaked onto an agar plate and incubated overnight, after a period of dark adjustment (approximately 10 to 15 min). Using this type of assay, West et al. (13) reported that 10% of a group of 115 *V. cholerae* isolates were luminescent. When several of the nonluminescent strains of the same group of strains were reevaluated by Palmer and Colwell (10), using a more sensitive method, liquid scintillation counting (chemilumines-

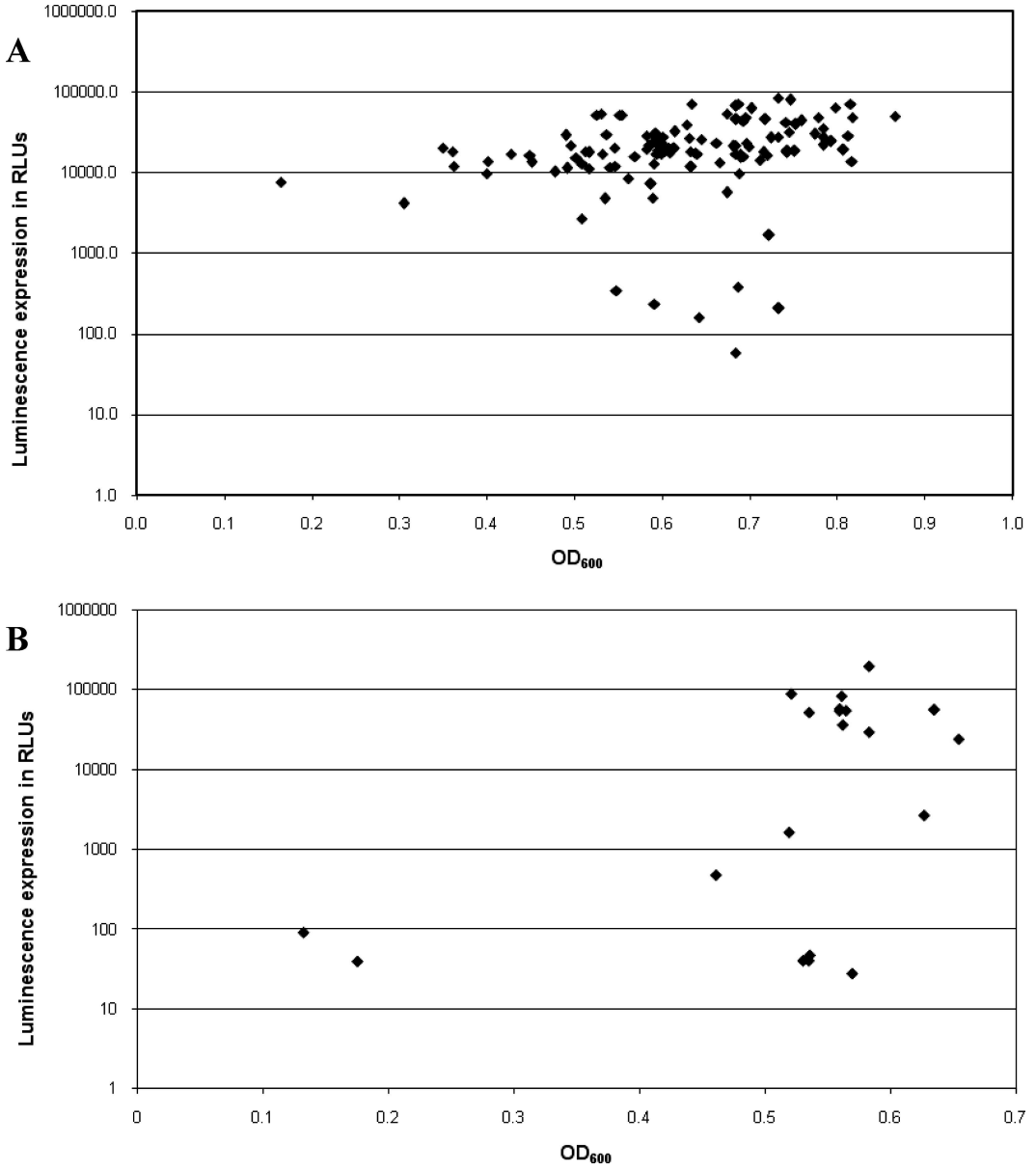

FIG. 3. Distribution of luminescent *V. cholerae* from Chesapeake Bay (group II) (A) and Bangladesh (group V) (B) by luminescence expression level compared to cell density at time of measurement (OD_{600}). RLU is defined in Materials and Methods.

cent mode), and employing 6-h broth cultures, they found that 21% of the nonluminescent strains were dimly luminescent.

In this study, an improved method for determining bioluminescence expression by *V. cholerae* was developed using mid- to late-exponential-phase broth cultures, grown in MB, and a luminometer for detection. Not surprisingly, mid- to late-exponential-phase broth cultures were most effective for assaying luminescence expression, which is a quorum-sensing controlled phenotype, as shown in Fig. 4A to C.

A total of five media were tested and MB was concluded to be the most effective for detecting luminescence, with a significantly greater expression of luminescence (Table 2). This result was confirmed when 47 of the 62 strains of *V. cholerae* that had been examined in the earlier study by Palmer and Colwell (10), were retested using our luminescence bioassay. MB (67% sensitivity) was found to be 50% more effective than LB medium (33% sensitivity) for detection of luminescence (Table 2). In addition, the intensity of luminescence in LB broth was significantly less than that in MB.

Not surprisingly, expression of luminescence by *V. cholerae* UM4102, UM4086, and UM4057 was observed to be stimulated by oxygen. The optimum ratio of air to liquid medium was 5:1 (tubes) to 6:1 (flask). Interestingly, luminescence expression decreased from maximum at ratios promoting the highest growth rates (15:1 and 10:1).

The luminometer was able to monitor luminescence in the range of 20 to 2,000,000 RLU, with a background of 20 RLU for uninoculated medium or nonluminescent *E. coli* DH5α. In

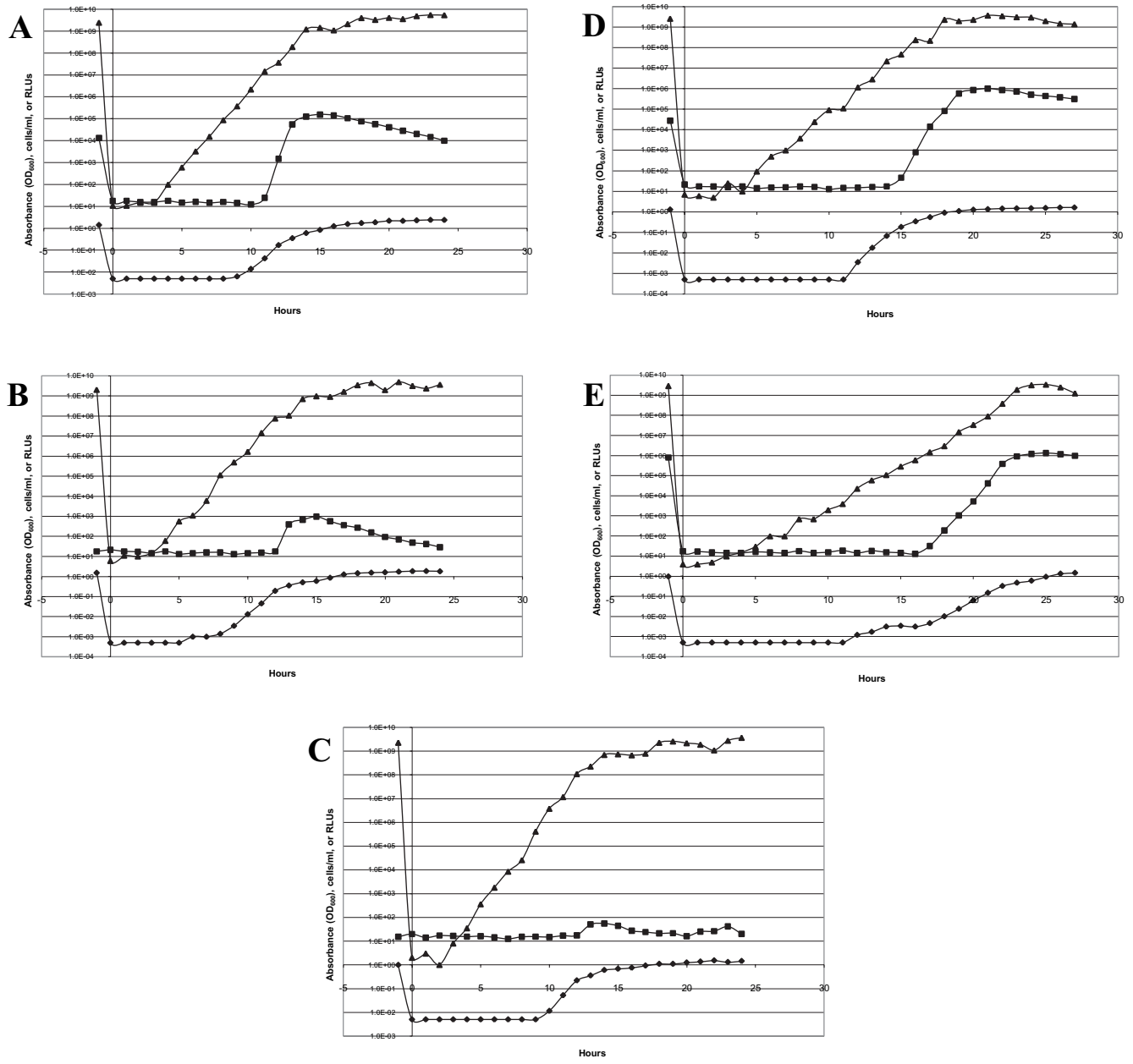

FIG. 4. Growth-luminescence kinetic curves for *V. cholerae* UM4089 (A), *V. cholerae* UM4086 (B), *V. cholerae* UM4103 (C), *V. fischeri* MJ100 (D), and *V. harveyi* BB120 (E). Cell density is shown by cell plate counts (cells/ml; triangles) and OD_{600} (diamonds), and luminescence is shown as RLU (squares).

comparison, the study by Palmer and Colwell (10), employing liquid scintillation counting, exhibited a range of RLU values of only 6.2, with a minimum value of 5.6 (Table 3). This lack of specificity resulted in five false-positives, UM4097, UM4100, UM4104, UM4105, and UM4178 (Table 3), in the previous study.

In this study, a high percentage (52%) of luminescent strains of *V. cholerae* isolated from the Chesapeake Bay, MD, an estuarine environment (group II), was observed. This proportion of luminescent strains was higher than that previously reported for *V. cholerae* (10%) (13). This result is explained primarily by the greater sensitivity of the luminescence bioassay. Additionally, group II (and III) strains were isolated from samples collected at regular sampling times in surveys of the seasonal and geographical distribution of *V. cholerae* in Chesapeake Bay, MD (group II), and rural Bangladesh (group III). The 47 strains of group I were, however, a laboratory collection of *V. cholerae* isolates from Louisiana (27 isolates), Chesapeake Bay (6), Tilamook Bay, Oregon (12), Florida (1), and England (1).

The *lux* structural operon was not present in the *Vibrio cholerae* O1 or O139 serogroup strains examined in this study, whether clinical strains from sources around the world (groups IV and V) or environmental strains from Bangladesh (group

III), yet the *lux* regulatory genes are present in the *V. cholerae* O1 strain that has been sequenced (5). This observation could be interpreted as suggesting either a significant evolutionary divergence between pathogenic and nonpathogenic strains of *V. cholerae*)if a common luminescent ancestor is assumed) or lateral transfer of genes to be a common mechanism for vibrios, especially when attached to chitinous surfaces (8). The *lux* operon also was not present in the 17 strains of the closely related species *V. mimicus* that were examined in this study.

A comparison of strains of *V. cholerae* isolated from a region of Bangladesh where cholera is endemic (group III, 6%) and from the Chesapeake Bay, an area where cholera is not endemic (group II, 52%), suggests that habitat and species diversity may influence the incidence of the luminescent phenotype. The occurrence of cholera in the Chesapeake Bay subsided in the early 20th century when sanitation and water treatment systems were installed. Nevertheless, *V. cholerae*, an estuarine bacterium, is a normal component of the Chesapeake Bay without the occurrence of epidemic cholera. In Bangladesh, neither appropriate sanitation nor safe drinking water systems are available to remote villages, and therefore cholera epidemics persist. In subsequent work, we will address this finding by analyzing strain diversity and examining environmental parameters.

In addition to the occurrence of luminescence, the pattern of luminescence expression also was different between the isolates from the Chesapeake Bay, MD, and Bangladesh. The majority (76%) of the luminescent strains of *V. cholerae* isolated from the Chesapeake Bay, MD, expressed light at an intensity characterized as "normal" for *V. cholerae*, namely, 10^4 to 10^5 RLU using this bioassay, although greater intensity was observed for 2 of the 875 strains used in this study. Only 1 of 116 (0.8%) Chesapeake Bay isolates was classified as "dim," and 16 isolates (14%) had some degree of defect in their luminescence expression. In contrast, luminescent strains from Bangladesh yielded a distribution that was bimodal, with 52% expressing luminescence at normal levels and 29% being classified as "dim."

In this study, we found that "dark" or K variants of luminescent *V. cholerae* occur commonly in nature (Fig. 2). Group I, screened using *luxA* PCR, contained 4 (8.5%) $luxA^+$ strains of *V. cholerae* that were not luminescent, while group II contained 14 (6.3%) and group III also contained 14 (4.2%). These strains can be explained by mutations in the *lux* operon or altered or defective regulation, but the exact cause for each strain was not investigated in this study. These strains account for the lack of sensitivity in the bioassay (67% sensitivity). Altered formulations of MB and additives reported to enhance luminescence expression were tested but failed to induce any detectable luminescence expression in these strains (data not shown).

Maximal expression of luminescence in *V. cholerae* was found to be, on average, 10% less than that in *V. harveyi* and *V. fischeri* (Fig. 4). Additionally, the rate of expression of luminescence, i.e., time of onset of luminescence to maximal expression, was higher in *V. cholerae* than in *V. harveyi* and *V. fischeri* (Fig. 4A and B compared to 4D and E).

Czyz et al. (4) showed that *lux* mutant strains are dominant to lux^+ strains of *V. harveyi* under normal growth conditions. Conversely, lux^+ strains of *V. harveyi* became dominant to *lux* mutant strains in cultures exposed to stress and damage induced by low levels of UV irradiation. It has been proposed that expression of the luciferase gene may help facilitate the bacterial DNA repair process by providing an internal light source for photoreactivation, a photo-mediated repair mechanism (4, 12). Alternatively, when attached to larger particles, such as detritus or plankton, *V. cholerae* may be afforded protection from UV damage, as would occur in Bangladesh ponds where turbidity is high (1). Further investigation of the functionality of the *lux* operon for the apparently nonsymbiotic but commensal species *V. cholerae* is needed, since the phenotype occurs more frequently than previously determined and demonstrates a dynamic expression range.

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